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Altered Serum Levels of Sex Steroids and Biotransformation Enzyme Activities by Long-term Alachlor Exposure in Crucian Carp (*Carassius auratus*)

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In the past several decades, a variety of environmental chemicals such as pesticides have been found to be responsible for detrimental reproductive effects in wildlife and human. These compounds, termed as endocrine disruptors (EDs), can alter endocrine function and subsequently may disrupt growth, development and reproduction (Colborn et al. 1993). Alachlor (Fig. 1) [2-chloro-*N*-(2, 6-diethyl phenyl)-*N*-(methoxymethyl) acetamide] is a commercialized effective pre-emergence and post-emergence herbicide in control of most grasses, most annuals and some broad leaved plants (Hayes and Laws 1991). It is a selective systemic herbicide, absorbed by germinating shoots and roots, works by interfering with the ability of plant to produce protein and by interfering with root elongation process. It has been used in the United States, Europe, Asia and other places in the world (Donaldson et al. 2002). Because of its widespread use, alachlor has been found in both surface water and groundwater in different countries (Barceló et al. 1996; Spalding et al. 2003). And the presence of alachlor in freshwater is likely to pose health hazards to non-target aquatic organisms and human (Chesters et al. 1989). Therefore, the aquatic ecological risk from this herbicide becomes an important worldwide concern (Gammon et al. 2005). Alachlor is classified as the carcinogen of B2 group and known as a highly toxic EDs (USEPA 1985). The potential toxicity of alachlor has been studied in a series of rodent chronic bioassays in mouse, rat, monkey, and isolated hepatocytes (Bonfant et al. 1992; Meisner et al. 1992). However, there has been a limited investigation of its endocrine disrupting effect in fish, which may be directly affected by this herbicide as they live in the surrounding aquatic environment near farmland. So far, only few studies have yet been performed by using crucian carp as an environmentally sensitive freshwater species.

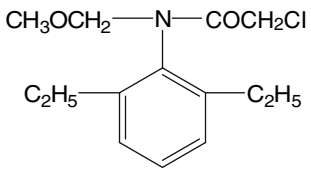

Fig. 1 Chemical structure of alachlor

The aim of this study is to investigate the endocrine disrupting effect including perturbation of the endocrine parameters and phase II biotransformation enzymes activities in response to long-term alachlor exposure. The serum hormonal levels of serum sex steroids (testosterone and 17β-estradiol) and activities of hepatic microsome enzymes (glutathione *S*-transferase (GST) and UDP-glucuronosyltransferase (UDPGT) are evaluated. The relationship between the levels of serum sex steroids and hepatic microsome enzyme activities is discussed.

Material and Methods

Alachlor (purity, 99.0%) was obtained from Jiangshan Pesticide Company of China (Jiangsu, People's Republic of China). All the other chemicals were analytical grade reagents, purchased from Sigma (St Louis, MO, USA) or chemical companies in China.

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Juvenile male crucian carp *Carassius auratus* (33.5 ± 7.6 g, 10.5 ± 1.0 cm) were purchased from Shanghai Fisheries Research Institute (Shanghai, People's Republic of China). All experimental fish were cultured in dechlorinated water (temperature 18 ± 5°C, pH 7.2 ± 0.3, DO 7.0 ± 0.4 mg/L) with a 12 h light:12 h dark regime for a minimum of 2 weeks prior to use, and fed with frozen adult brine shrimp every morning.

Then crucian carp were continuously water-borne exposed to alachlor in a series of concentrations (1, 4, 16, 63, 250 and 500 μg/L) for 60 days, with an untreated group for control. In this study, fish were randomly assigned into 7 study groups. For each treatment and control, 5 fish were placed in a 50 L glass aquarium in five replicates, giving a total of 25 animals per treatment and control. No fish died in the experiment periods. The experiment was carried out using a static-renewal regimen, half of the exposure solution was renewed daily to ensure the stabilization of test substance concentrations. Alachlor was measured by gas chromatograph with electron capture detector (Ramesh and Maheswari 2004).

After 60 days of exposure, fish were anesthetized on each sampling occasion, the blood was collected from the caudal vessel with heparinized syringes. To prevent proteolysis, 4 TIU/mL aprotinin was added, the blood was centrifuged at 5,000*g* for 30 min at 4°C, serum was collected and stored at −70°C. Gonads and livers of crucian carp were carefully dissected from adipose tissue and pancreas, rinsed with physiological salt water, and weighed. Then the livers were placed into ice-cold 0.25 M sucrose solution containing 10 mM Tris-HCl buffer, pH 7.5, and 0.1 mM EDTA and homogenized with 9 volume of the same sucrose medium in a Potter–Elvehjem glass homogenizer using a glass pestle. The homogenates were centrifuged at 9,000 rpm for 20 min at 4°C. The initial supernatant was carefully decanted and recentrifuged at 105,000*g* for 60 min to pellet the microsome. The surface of each microsomal pellet was washed with cold 0.25 M sucrose, and finally microsomal pellet was then resuspended in 1 mL of cold 0.25 M sucrose by homogenizing 15 s on Polytron at a setting of 4. The microsomes were stored at −70°C for enzyme activity analysis.

The serum levels of sex steroids (testosterone and 17β-estradiol) levels were measured using radioimmunoassay (RIA) assay (^{125}I RIA kits, Tianjin Jiuding Parmaceutic and Bioengineering Ltd, Tianjin, China). For the actual measurements a Gamma Counter (1470 Wizard; Wallac, Turku, Finland) was used.

GST activity was measured according to Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. Assays were performed in a reaction mixture containing 1.05 mL of 100 mM Tris buffer (pH 7.4), 50 μl CDNB (1 mM), 50 μl GSH (1 mM) and 50 μL tissue homogenate. The required GSH for the assay was dissolved in Tris buffer beforehand. The CDNB was dissolved in ethanol. In all cases, the final concentration of ethanol in the assay mixture did not exceed 5% (*v*/*v*). Blanks had the same conditions replacing the sample with Tris buffer. Enzyme activity was determined by monitoring changes in absorbance at 340 nm for 2 min at constant temperature with a UV-2100 spectrophotometer. The GST activity was expressed as μmol/min/mg protein. UDPGT activity was measured using a method as described by Owens (1977). The assay mixture contained 150 μL 45 mM UDP glucuronic acid, 1.5 μL 0.35 M p-nitrophenol, 30 μL 500 mM EDTA, and 818.5 μL 1.0 M K-phosphate buffer (pH 6.5) in a total volume of 1 mL. Microsome sample of 50 and 100 μL assay mixture were incubated at 25°C and terminated after 20 min with 0.9 mL of 3% trichloroacetic acid (TCA). After centrifugation for 5 min at 1,200 rpm, the supernatant was treated with 1 M NaOH and the amount of remaining *p*-nitrophenol was measured at 405 nm using Bio-Rad Model 3550 plate reader. UDPGT activity was expressed as μmol/min/mg microsomal protein. The microsome protein levels of liver samples were spectrophotometrically measured by the method of Bradford (1976) using bovine serum albumin as standard. Absorbances of samples were detected at 595 nm.

Results in this paper were expressed as mean ± SD and analyzed using the SPSS for Windows 10.0 program. When data did not follow a normal distribution, it was log 10-transformed prior to the analysis, to achieve homogeneity of variance. Significant differences between means were performed with ANOVA and followed by the post hoc Duncan's Multiple Range test for subsequent comparisons between control and treated groups. Differences were considered significant at $p < 0.05$. Asterisks in figures indicated values that were significantly different from control at $p < 0.05$.

Results and Discussion

After 60 days alachlor exposure, there were no differences in the level of body length and body weight of all groups (data not shown). But significant decreases ($p < 0.05$) of gonadosomatic index (GSI) and hepatosomatic index (HSI) were observed in almost all treatment (see Figs. 2, 3). GSI and HSI could be a simple toxic effect indicator of pollution exposure since they are easy parameters to measure.

Sex steroids such as testosterone and 17β-estradiol regulate the gonad development, yolk formation, and oocyte maturation in fish. Observation of the alteration of serum levels of sex steroids in fish may act as a useful method to screen the EDs in aquatic environment and may be a sensitive index to evaluate the functional damage of

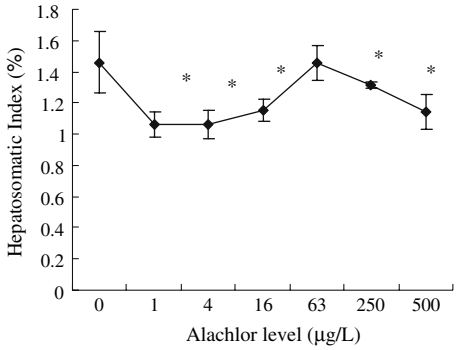

Fig. 2 HSI of juvenile crucian carp after 60 days exposure to alachlor

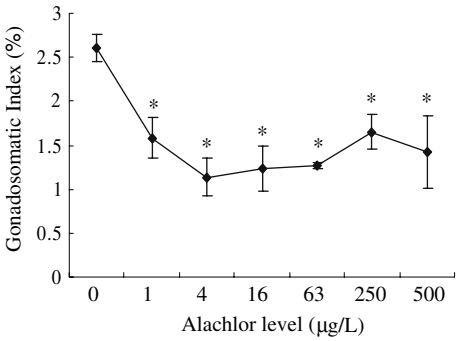

Fig. 3 GSI of juvenile crucian carp after 60 days exposure to alachlor

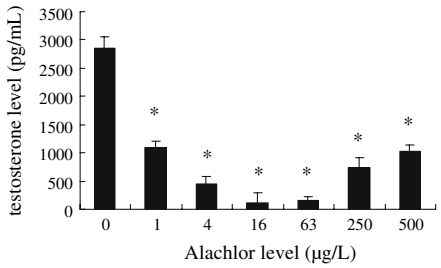

Fig. 4 Effect of alachlor on the testosterone levels in crucian carp serum

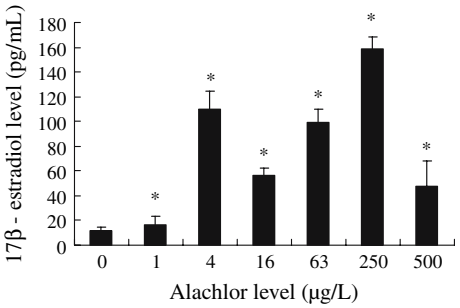

Fig. 5 Effect of alachlor on the 17β-estradiol levels in crucian carp serum

reproduction in fish (Ankley et al. 1998). The concentrations of both testosterone and 17β-estradiol in the serum of crucian carp after 60 days exposure to alachlor were measured, and significant changes in sex hormones were observed.

As shown in Fig. 4, alachlor caused a dose-dependent decrease in testosterone level ($p < 0.05$). Moreover, it gave a biphasic effect on serum testosterone levels. The testosterone levels were continuously inhibited at 1, 4 and 16 μg/L treatments. Then slight increases of testosterone were observed while the concentrations were still lower than the control group. A maximum inhibition of 96% was observed at 16 μg/L alachlor-treated group. The 17β-estradiol levels were significantly increased ($p < 0.05$) at alachlor-treated groups with large variation (Fig. 5). The maximum increase in 17β-estradiol level was approximately 1,200% to that of control and was observed at 250 μg/L group. These data indicated the overall effects of long-term alachlor exposure on circulating steroid concentrations. And further studies are needed to elucidate the mechanisms underlying how pollutants exert their effects.

Enzymatic biotransformation is an important process responsible for the detoxification and elimination of EDs. And certain biotransformation phase I and II enzymes could regulate and control sex steroid hormones levels in fish (Andersson and Forlin 1992). The enzymes involved in biotransformation phase II step are usually transferases such as GST and UDPGT. These enzymes play a key role in the metabolism and protecting against the toxic effects of EDs (Hayes and Pulford 1996). This property makes them suitable for pollution monitoring in the aquatic environment.

The significant response in hepatic GST and UDPGT activities in crucian carp were determined. As shown in Fig. 6, GST activities were significantly induced ($p < 0.05$) at 4, 16, 63 and 500 μg/L treatments. And 250 μg/L group was lower than 63 and 500 μg/L. A maximum increase of 44% and a maximum inhibition of 12% were observed at 63 and 250 μg/L, respectively. Then a rebound was observed at the highest concentration treatment with a 39% increase to the control group. GST levels could be increased due to an adaptive mechanism to slight oxidative stress through an increase in its synthesis; however, a severe oxidative stress may suppress GST levels due to the exhaustion of GSH (Zhang et al. 2005). The UDPGT activity showed a quite clear inhibition ($p < 0.05$) due to the accumulation of alachlor exposure (Fig. 7). Levels of UDPGT activity were decreased 66% compared to control levels. This continuous suppression should be due to a nonspecific toxic effect of alachlor with the increasing relatively high dosages.

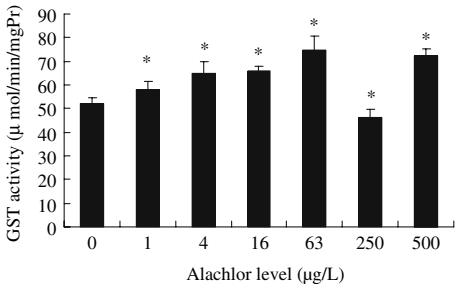

Fig. 6 Effect of alachlor on the activities of hepatic GST in crucian carp livers

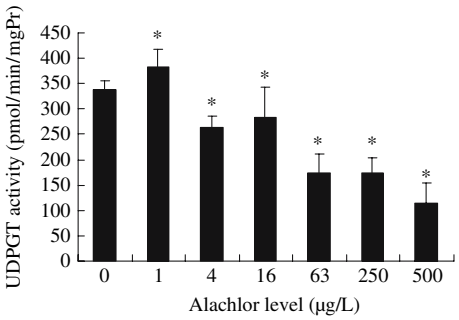

Fig. 7 Effect of alachlor on the activities of hepatic UDPGT in crucian carp livers

The results in the present study indicated that GST and UDPGT probably contributed to the change of serum sex steroid levels in crucian carp and most likely will have negative consequences on reproduction (Qian et al. 2004). In this study, there was also a significant negative correlation between the liver GST activities and the serum testosterone levels, although a xenobiotic disruption of the normal metabolic clearance pathways could not be ruled out. This indicated that the suppression of the circulating testosterone levels could be related to the increase in the GST activity in the crucian carp. In general, the increased serum 17β-estradiol levels in all alachlor treated groups could be explained by depression of hepatic biotransfomation enzymes, decreased steroid clearance and increased steroid biosynthesis. UDPGT which existing in the hepatic microsomes is important enzyme responsible for the hepatic degradation of sex steroids (Baldwin and Leblanc 1994). This is probably due to the role of UDPGT in the maintenance of steroid hormone levels in the organisms. UDPGT may be important for the conjugation and limination of 17β-estradiol, due to increasing its solubility in water. There have been several researches about UDPGT activity changes resulting from environmental toxicants exposure to fish. Reduced levels of plasma steroid with elevated UDPGT activity treatment of fish under PCB treatment were reported (Huggett et al. 1992). While some other environmental contaminants have been found to being inhibitive effects on hepatic UDPGT activities in rainbow trout like trichlorophol, pentachlorophenol which are common chemicals in pulp mill effluent (Andersson et al. 1988). From a mechanistic basis, the lower UDPGT activities in crucian carp were due, at least in part, to decreased levels of steady state hepatic UDPGT protein. Collectively, these data suggested that environmental stressors might have compromised the ability of fish to either synthesize or maintain hepatic UDPGT activities. The fact that crucian carp had elevated tissue burdens of persistent alachlor is suggestive of a role of environmental chemical exposure in the loss of UDPGT activities.

In summary, the results demonstrated that long-term administration of alachlor could affect multiple physiological systems. Significant decreases in GSI and HSI were observed. Results showed that alachlor significantly decreased serum testosterone levels in the crucian carp compared to the control, but 17β-estradiol levels were significantly increased with large variation, and hepatic microsome GST activities were continuously induced. Alachlor resulted in a marked inhibition to UDPGT activity at all doses. The alteration of Phase II metabolizing enzymes may account for the observed changes in serum sex steroids levels. These also demonstrated that the alachlor may have endocrine disrupting effect in crucian carp and may have profound effects on the reproductive success of this species and other fish.

From an ecotoxicological perspective, crucian carp is a desirable bioindicator species for screening the EDs in the aquatic environment in many countries where crucian carp exists as predominate freshwater species. However, the exclusive biological consequences of these alterations, in particular, the possible effects on the reproductive capacity of fish, are unclear and should be the subject of further study.

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